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Your Reference: JAF/pms/SBplc/P31672

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Request for grant of a Patent

Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention NOVEL COMPOUNDS

❷ Applicant's details

❑ **First or only applicant**

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4. Agent's or applicant's reference number *(if applicable)*

❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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Yes ☐ No ☒ ➲ *go to 6*

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

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If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number

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6

- any applicant is not an inventor
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8 *Please supply duplicates of claim(s), abstract, description and drawings).*

8 Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 ☐

Claim(s)	**2**	Description	**19**
Abstract	**-**	Drawing(s)	**-**

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)* ☐

Translation(s) of Priority documents *(please state how many)* ☐

Patents Form 7/77 - Statement of Inventorship and Right to Grant ☐

Patents Form 9/77 - Preliminary Examination Report ☐

Patents Form 10/77 - Request for Substantive Examination ☐

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9 Request

I/We request the grant of a patent on the basis of this application.

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Signed Julia Florence Date: 27 November 1996
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NOVEL COMPOUNDS

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is a novel human neurotrophic factor receptor and its splice variants. The polypeptide is especially an alpha receptor, in particular a Glial cell-line Derived Neurotrophic Factor (GDNF) alpha-like receptor. The invention also relates to modulating the action of such polypeptides and to the identification of drugs which modulate the actions of such polypeptides.

Glial cell-line Derived Neurotrophic Factor (GDNF) has been reported tobe a potent survival factor for central and peripheral neurons and to be essential for the development of kidneys and the enteric nervous system. The GDNF receptor comprises a binding receptor, termed the GDNF alpha receptor (Treanor, J. et al. (1996), Nature **382** 80-83 and Jing, S. et al. (1996), Cell **85** 1113-1124) and a signalling component, the tyrosine kinase receptor Ret (Trupp, M. et al. (1996), Nature **381** 785-789 and Durbec, P. et al. (1996), Nature **381** 789-793).

In accordance with an aspect of the present invention, there is provided a polypeptide which is a novel GDNF alpha-like receptor (herein referred to as GDNF alpha 3 receptor) polypeptide:

characterised by the deduced amino acid sequence of SEQ ID NO 2;

or a fragment, analogue or derivative thereof.

Hereinafter the term polypeptide(s) will be used to refer to the GDNF alpha 3 receptor and it fragments analogues and derivatives. The polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 2 is referred to herein as the GDNF alpha 3 receptor.

In accordance with another aspect of the invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides.

In accordance with a preferred aspect of the invention, there is provided a polynucleotide which encodes a polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 2.

In particular, the invention provides a polynucleotide comprising the partial DNA sequence given in SEQ ID NO 1.

The DNA sequence given in SEQ ID NO 1 is a partial sequence, which represents some 30-50% of the coding region of the full-length clone. It will be appreciated that the deduced amino acid sequence given in SEQ ID NO 2 is thus also a partial sequence, representing some 30-50% of the complete amino acid sequence. It is believed however

that both the partial DNA and amino acid sequences are sufficient to characterise the corresponding full length sequences. It will be appreciated therefore that the GDNF alpha 3 receptor according to the present invention is not represented in its entirety by SEQ ID NO 2, but includes and is characterised by this portion of the sequence.

Although the isolated human GDNF alpha 3 cDNA does not contain the entire open reading frame at the 5' end, a full length clone can easily be obtained by methods well known to persons skilled in the art. Among the alternative methods of choice, the following examples may be mentioned:

1) The human library type cDNA library can either be rescreened with a probe from the 5' end of the already cloned sequence,

2) Anchor-PCR or RACE (Rapid Amplificaiton of cDNA Ends) (Kriangkum et al., Nucleic Acids Res. 20 (1992) 3793 - 3794: Troutt et al., Proc. Natl Acad. Sci., USA 89 (1992), 9823 -9825) methodology can be used to clone the remaining 5' sequences from cDNA from a suitable tissue source.

3). The remaining 5' part can be isolated from human genomic libraries, and DNA fragments considered to represent introns can be identified by homology to the cDNA of the rat receptor and deleted by mutagenesis. Alternatively the 5' portion can be isolated from genomic DNA fragments, generated either by mechanical shearing (eg sonication) or enzymatic digestion. Such fragments include preparations where oligonucleotide anchors are ligated onto the ends to enable RACE-like reactions to be carried out as exemplified in, for example, the GenomeWalker kit (TM, Clontech).

The 5' end of the cDNA can be fused to the remaining 3' part of the human GDNF alpha 3 polypeptide cDNA by the use of PCR or through fusion at appropriate restriction enzyme recognition seqeuences identified in both the 5' and the 3' parts.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may comprise the coding sequence shown in SEQ ID NO 1or may comprise a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA comprising SEQ ID NO 1.

The polynucleotide which encodes for the mature polypeptide of the invention may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory

sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the mature polypeptide. Preferred embodiments of the invention are polynucleotides that are at least 70% identical to a polynucleotide encoding the polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 2, and polynucleotides which are complementary to such polynucleotides. Most highly preferred are polynucleotides that comprise a region that is at least 80% identical to a polynucleotide encoding the polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 2, and polynucleotides complementary thereto. In this regard, polynucleotides at least 90% identical to the same are particularly preferred, and those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred and those with at least 98-99% are most highly preferred, with at least 99% being the most preferred. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the mature polypeptide. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which comprises a naturally occurring allelic variant of the coding sequence shown in SEQ ID NO 1. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is

the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 or pET vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide of the invention.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The terms "fragment," "derivative" and "analogue" when referring to the mature polypeptide of the invention, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analogue includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analogue of the mature polypeptide of the invention may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example,

polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is therefore provided a process for producing the polypeptide of the invention by recombinant techniques by expresssing a polynucleotide encoding said polypeptide in a host cell and recovering the expressed product. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the human GDNFα 3 receptor genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids;

vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli (including AD494 and related strains that allow for the formation of disulphide bonds within the cytoplasm), Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The

following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer,

the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), _-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The polypeptide of the present invention is also useful for identifying other molecules which may have similar biological activity. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polypeptides may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being

provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

According to the invention there is provided a method for the treatment of a patient having need to modulate GDNF α 3 receptor activity, said method comprising administering a therapeutically effective amount of the polypeptide of the invention, for example by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes, or fragments thereof (as in radiation hybrid panels). Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other

mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. FISH requires use of the clones from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Alternatively, chromosomal location can be determined using Radiation Hybrid (RH) mapping. This method relies upon fragmentation of human chromosomes with X-rays, and retention of these random fragments in Hamster A23 host cells. The DNAs for RH mapping are supplied by Research Genetics (USA). Oligo pairs are designed from EST sequences that will amplify products of between 80bp and 300bp. The PCRs are performed on 93 human / hamster hybrid DNAs and the results compared with a framework map (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl, Gyapay, G et al (1996) Human Molecular Genetics 5 : 339-346. RH mapping provides greater precision than FISH and indicates clusters of genes as well as disease locus / gene correlations.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be

one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

The invention provides a method for screening for agonist compounds, which can trigger signalling by a relevant tyrosine kinase receptor eg Ret. As an example a mammalian cell or membrane preparation expressing GDNF alpha 3 receptor and the signalling tyrosine kinase receptor would be incubated with individual compounds (in the absence of GDNF or GDNF homologues) and agonist compounds identified by demonstration of their ability to stimulate autophosphorylation of the tyrosine kinase

receptor. This invention also provides a method of screening drugs to identify those which block (antagonists) functional interaction of ligand with the GDNF alpha 3 receptor of the invention, the method comprising measuring the ability of test compound to block interaction of ligand (usually GDNF or a homologue thereof) with the receptor. As an example, a mammalian cell or membrane preparation expressing the GDNF alpha 3 receptor would be incubated with labeled ligand in the presence of the drug. The ability of the drug to block this interaction could then be measured. An example of such an assay combines an appropriate ligand labelled with radio-activity, eg., [^{125}I]-GDNF or a homologue thereof, and a potential antagonist/inhibitor with membrane-bound GDNF alpha 3 receptors or recombinant forms of the GDNF alpha 3 receptor under appropriate conditions for a competitive inhibition assay, such that the number of radiolabelled molecules bound to the receptor can determine the effectiveness of the potential antagonist/inhibitor. Alternatively, the response of a known second (or third) messenger system following interaction of ligand and receptor would be measured compared in the presence or absence of the drug. Such messenger systems include but are not limited to, tyrosine kinase cascades, as measured by protein phosphorylation of pathway components.This invention also provides transgenic non-human animals comprising a polynucleotide encoding a polypeptide of the invention. Also provided are methods for use of said transgenic animals as models for mutation and SAR (structure/activity relationship) evaluation as well as in drug screens.

The present invention is also directed to agonist molecules of the polypeptides of the present invention, and their use in mimicking the function of the polypeptide.

The invention further provides a method for the treatment of a patient having need to enhance the function of the polypeptide comprising administering to the patient a therapeutically effective amount of the agonist of the invention.

An example of an agonist is a small molecule which binds to and occupies the receptor binding site, whilst mimicking the activity of the naturally occurring ligand. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The present invention is also directed to antagonist/inhibitor molecules of the polypeptides of the present invention, and their use in reducing or eliminating the function of the polypeptide.

The invention further provides a method for the treatment of a patient having need to reduce or eliminate the function of the polypeptide comprising: administering to the patient a therapeutically effective amount of the antagonist/inhibitor of the invention.

An example of an antagonist is an antibody or in some cases, an oligonucleotide which binds to the polypeptide. The antagonists may be closely related proteins such that they recognize and bind to the interacting components of the natural protein, however, they are inactive forms of the polypeptide and thereby prevent signalling via the Ret receptor .

An example of an inhibitor is an antisense constructs prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of the mature polypeptide. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the GDNF α3 receptor (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the GDNF α3 receptor.

Another example of an antagonist/inhibitor is a small molecule which binds to and occupies the receptor binding site, thereby making the site inaccessible to its natural ligand or which binds to a related site which affects the function of GDNF or homologues in an allosteric manner such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The GDNF α3 receptors of the invention have been found to be present in certain tissues, such as the skeletal muscle and testes, where they will play a role in cell survival in various conditions associated with tissue damage - eg., CNS and peripheral neuropathies.

The polypeptides, polynucleotides and antagonist/inhibitors of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions, which form part of the invention, comprise a therapeutically or prophylactically effective amount of the active substance, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline,

buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the active substances of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The active substance is administered in an amount which is effective for treating and/or prophylaxis of the specific indication. The amounts and dosage regimens of active substance and administered to a subject will depend on a number of factors such as the mode of administration, the nature of the condition being treated and the judgment of the prescribing physician. In general, the active substance will be administered in an amount in the range of at least about 0.1-100μg/kg body weight per day, taking into account the routes of administration, symptoms, etc.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for

particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37 C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

Example 1

Isolation of GDNF α3 cDNA clones and DNA sequencing

An EST sequence was identified by homology to the published rat GDNF α receptor sequence, from an adult testis cDNA library. The sequence of the clone from which the EST was derived was determined using standard sequencing techniques (Sequenase (TM) Amersham) giving the partial coding region up to the stop codon and the 3' untranslated region. The partial coding region is shown in SEQ ID NO 1 below.

Example 2

Tissue localization

Initial tissue localisation was carried out using the Clontech Quickscreen panel. The expected band size for reactions involving this primer pair is 69bp, and a very strong band of this size was recovered in the skeletal muscle template reaction.- Additional localisation in cDNA libraries has identified the sequence in testes.

Example 3

Chromasomal localization

Using radiation hybrid mapping the GDNF α3 gene has mapped to 5q31.

Figure 1 - Partial coding cDNA sequence for human GDNF α3 receptor
SEQ ID NO 1

```
1    GAGCGCCGGC GCAACACCAT CGCCCCCAAC TGCGCGCTGC CGCCTGTGGC
51   CCCCAACTGC CTGGAGCTGC GGCGCCTCTG CTTCTCCGAC CCGCTTTGCA
101  GATCACGCCT GGTGGATTTC CAGACCCACT GCCATCCCAT GGACATCCTA
151  GGAACTTGTG CAACAGAGCA GTCCAGATGT CTACGAGCAT ACCTGGGGCT
201  GATTGGGACT GCCATGACCC CCAACTTTGT CAGCAATGTC AACACCAGTG
251  TTGCCTTAAG CTGCACCTGC CGAGGCAGTG GCAACCTGCA GGAGGAGTGT
301  GAAATGCTGG AAGGGTTCTT CTCCCACAAC CCCTGCCTCA CGGAGGCCAT
351  TGCAGCTAAG ATGCGTTTTC ACAGCCAACT CTTCTCCCAG GACTGGCCAC
401  ACCCTACCTT TGCTGTGATG GCACACCAGA ATGAAAACCC TGCTGTGAGG
451  CCACAGCCCT GGGTGCCCTC TCTTTTCTCC TGCACGCTTC CCTTGATTCT
501  GCTCCTGAGC CTATGGTAG
```

Figure 2 - Deduced amino acid sequence for the partial GDNF α3 receptor sequence SEQ ID NO 2:

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1    ERRRNTIAPN CALPPVAPNC LELRRLCFSD PLCRSRLVDF QTHCHPMDIL
51   GTCATEQSRC LRAYLGLIGT AMTPNFVSNV NTSVALSCTC RGSGNLQEEC
101  EMLEGFFSHN PCLTEAIAAK MRFHSQLFSQ DWPHPTFAVM AHQNENPAVR
151  PQPWVPSLFS CTLPLILLLS LW
```

The sequence underlined corresponds to the predicted hydrophobic C-terminus characteristic of GPI anchored cell-surface receptors

Claims

1. A polynucleotide encoding a polypeptide which is a human GDNF α3 receptor polypeptide, said polypeptide being characterised by the deduced partial amino acid sequence of SEQ ID NO 2; or a fragment, analogue or derivative thereof.

2. The polynucleotide of claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of claim 2 wherein the polynucleotide is genomic DNA.

5. The polynucleotide of claim 2 comprising the partial DNA sequence given in SEQ ID NO 1.

6. The polynucleotide of any preceding claim in isolated form.

7. A vector containing the DNA of any one of claims 2, 4, 5 or 6.

8. A host cell genetically engineered with the vector of claim7.

9. A process for producing a polypeptide comprising expressing from the host cell of claim 8 the polypeptide encoded by said DNA.

10. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of claim 7.

11. A polynucleotide hybridizable to the polynucleotide of any one of claims 1 to 6 and encoding a polypeptide having substantially the same biological function or activity as the polypeptide characterised by the deduced amino acid sequence of SEQ ID NO 2

12. A human GDNF α3 receptor polypeptide characterised by the deduced partial amino acid sequence of SEQ ID NO 2;
or a fragment, analogue or derivative thereof.

13. The polypeptide of claim 12 in isolated form.

14. A method of screening drugs to identify those which mimic or those which block functional interaction of ligand with the human GDNF α3 receptor of claim 12, the method comprising measuring the ability of test compound to block interaction of ligand with the receptor.

15. A compound identified by the method of claim 14.

16. An agonist or antagonist/inhibitor of the polypeptide of claim 12.

17. A pharmaceutical composition comprising the polynucleotide of claim 1 or 11, a polypeptide of claim 12, a compound of claim 15 or an agonist or antagonist of claim 16 and a pharmaceutically acceptable carrier.

20. A method for the treatment of a patient having need to enhance the function of the polypeptide of claim 12 comprising administering to the patient a therapeutically effective amount of an agonist of claim 16.

21. A method for the treatment of a patient having need to reduce or eliminate the function of the polypeptide of claim 12 comprising administering to the patient a therapeutically effective amount of an antagonist/inhibitor of claim 16.

22. The use of a compound of claim 15 or an agonist or antagonist/inhibitor of claim 16 for the manufacture of a medicament for use in therapy.

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